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Chromosomal instability by mutations in the novel minor spliceosome component *CENATAC*

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Abstract

Aneuploidy is the leading cause of miscarriage and congenital birth defects, and a hallmark of cancer. Despite this strong association with human disease, the genetic causes of aneuploidy remain largely unknown. Through exome sequencing of patients with constitutional mosaic aneuploidy, we identified biallelic truncating mutations in *CENATAC* (*CCDC84*). We show that CENATAC is a novel component of the minor (U12-dependent) spliceosome that promotes splicing of a specific, rare minor intron subtype. This subtype is characterized by AT-AN splice sites and relatively high basal levels of intron retention. CENATAC depletion or expression of disease mutants resulted in excessive retention of AT-AN minor introns in ~100 genes enriched for nucleocytoplasmic transport and cell cycle regulators, and caused chromosome segregation errors. Our findings reveal selectivity in minor intron splicing and suggest a link between minor spliceosome defects and constitutional aneuploidy in humans.

Keywords aneuploidy; CCDC84; CENATAC; minor spliceosome

Subject Categories Cell Cycle; Genetics, Gene Therapy & Genetic Disease; RNA Biology

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Introduction

Chromosome segregation errors in mitosis or meiosis lead to aneuploidy, a karyotype that deviates from an exact multiple of the haploid set of chromosomes. Aneuploidy is the leading cause of congenital birth defects and associated with ~35% of all spontaneous human abortions (Nagaoka *et al,* 2012). Furthermore, roughly 70% of human tumors are aneuploid, making it one of the most common genomic alterations in cancer (Duijf & Benezra, 2013; Knouse *et al,* 2017). Despite this common association of aneuploidy with human disease, little is known about its genetic causes. The study of aneuploidy-associated hereditary disorders can be instrumental in uncovering these causes.

Mosaic variegated aneuploidy (MVA; OMIM: 257300) is a rare autosomal recessive disorder characterized by mosaic aneuploidies in multiple tissues. Patients often present with microcephaly, developmental delay, various congenital abnormalities, and childhood cancers (García-Castillo *et al,* 2008). Pathogenic mutations in *BUB1B*, *CEP57*, or *TRIP13*, have been identified in roughly half of all MVA patients (Hanks *et al,* 2004; Matsuura *et al,* 2006; Snape *et al,* 2011; Yost *et al,* 2017). These genes have well-documented roles in chromosome segregation (Suijkerbuijk *et al,* 2010; Sacristan & Kops, 2015; Vader, 2015; Zhou *et al,* 2016). All three gene products (BUBR1, CEP57, and TRIP13) promote spindle assembly checkpoint (SAC) function (Wang *et al,* 2014; Musacchio, 2015; Ma *et al,* 2016; Zhou *et al,* 2016; Alfieri *et al,* 2018), and BUBR1 and CEP57 additionally ensure correct kinetochore–microtubule attachment (Emanuele & Stukenberg, 2007; Sacristan & Kops, 2015). As predicted, such mitotic processes are defective in cells from MVA

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patients carrying biallelic mutations in these genes, explaining the chromosomal instability (CIN) phenotype and resulting aneuploid karyotypes. CIN can also result from mutations in regulators of expression of mitotic genes. For example, mutations in the retinoblastoma gene (*RB1*) cause CIN by overexpression of the SAC protein MAD2 (Hernando *et al,* 2004; Sotillo *et al,* 2007; Schvartzman *et al,* 2011). In this work, we show that chromosome segregation errors can be caused by a specific defect in minor intron splicing, another process governing correct gene expression.

While the conventional, major spliceosome targets most (> 99.5%) human introns, the minor spliceosome recognizes and excises only a small subset (~ 700 introns) (Turunen *et al,* 2013a; Moyer *et al,* 2020). These minor introns (also called U12-type introns) have highly conserved 5′ splice site (5′ss) and branch point (BPS) sequences that are longer and differ at the sequence level from the respective sequences in major (U2-type) introns. Most minor introns have AT-AC or GT-AG terminal dinucleotides (24 and 69%, respectively) (Sheth *et al,* 2006; Moyer *et al,* 2020). In addition, the 3′ terminal nucleotide can vary, thus giving rise to AT-AN and GT-AN classes of minor introns (Levine & Durbin, 2001; Dietrich *et al,* 2005). For simplicity, we refer to these as A- and G-type introns, respectively. Thus far, there has been no indication of mechanistic or functional differences between the minor intron subtypes.

Minor intron "host" genes, the position of the minor intron within the gene, and intron subtypes, are all evolutionarily conserved (Burge *et al,* 1998; Abril *et al,* 2005; Sheth *et al,* 2006; Alioto, 2007; Moyer *et al,* 2020). Despite this high conservation, the functional significance of minor introns has remained elusive. Elevated levels of unspliced minor introns in various cell types have been reported, giving rise to the hypothesis that these are rate-limiting controls for the expression of their host genes (Patel *et al,* 2002; Younis *et al,* 2013; Niemelä & Frilander, 2014; Niemelä *et al,* 2014). Nevertheless, the overall significance of the elevated intron retention (IR) levels has been questioned particularly at individual gene level (Singh & Padgett, 2009).

The overall architecture of the minor and major spliceosomes is highly similar. Both are composed of five small ribonucleoprotein (snRNP) complexes containing small nuclear RNA (snRNA) molecules and a large number of protein components. One of the snRNAs (U5) is shared between the spliceosomes, while U1, U2, U4, and U6 snRNAs are specific to the major spliceosome, and U11, U12, U4atac, and U6atac snRNAs to the minor spliceosome. Introns are initially recognized by the U1 and U2 snRNPs (major spliceosome) or by the U11/U12 di-snRNP (minor spliceosome), followed by the entry of the U4/U6.U5 or U4atac/U6atac.U5 tri-snRNP and subsequent architectural changes leading to catalytic activation of the spliceosome (Turunen *et al,* 2013a). At the protein level, the main difference between the spliceosomes is in the composition of the U11/U12 di-snRNP that contains seven unique protein components that are needed for recognition of the unique minor intron splice sequences (Will *et al,* 2004). In contrast, the protein composition of the minor and major tri-snRNPs appears similar, but rigorous comparative analyses have been difficult due to the ~ 100-fold lower cellular abundance of the minor tri-snRNP (Schneider *et al,* 2002).

Here, we report that germline mutations in a novel component of the minor spliceosome (*CENATAC*/*CCDC84*) cause chromosomal instability in MVA patients. We identify CENATAC as a minor spliceosome-specific tri-snRNP subunit that promotes the splicing of A-type minor introns, but hardly contributes to G-type minor intron splicing. We show that CENATAC depletion or disease mutations result in increased A-type minor IR and mitotic chromosome congression defects. Congression defects are also seen when another minor spliceosome component is depleted, suggesting that the chromosome segregation errors and aneuploidy observed in MVA patient cells are secondary effects of defective minor intron splicing.

Results

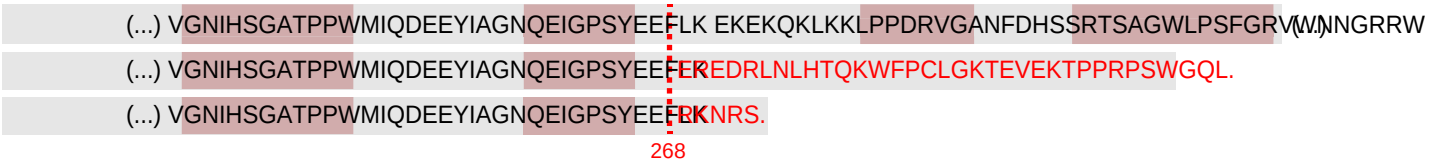
Biallelic truncating mutations in *CENATAC* (*CCDC84*) cause MVA

To search for additional causes of MVA, we performed exome sequencing and variant analyses on MVA patients and family members, as previously described (Yost *et al,* 2017). We identified biallelic truncating mutations in coiled-coil domain-containing 84 (*CCDC84,* hereafter named *CENATAC*, for centrosomal AT-AC splicing factor, see below) in two affected siblings with 7.3 and 8.5% aneuploid blood cells, respectively (Figs 1A and EV1). Both siblings were alive at 47 and 33 years of age and had microcephaly, mild developmental delay, and mild maculopathy. Neither individual had short stature, dysmorphism, or cancer. Each parent was heterozygous for one of the mutations, and the unaffected sibling had neither mutation. Moreover, the mutations were absent from the ExAC and ICR1000 series and we estimated the chance of an individual having two truncating *CENATAC* mutations to be 4.8×10^{-10} (Fitzgerald *et al,* 2015). We therefore consider it very likely that the *CENATAC* mutations are the cause of the siblings' phenotype. The paternal and maternal mutations (mutation 1 and mutation 2, respectively) both result in the creation of novel splice sites that lead to a frameshift and the loss of the C-terminal 64 amino acids of CENATAC (Fig 1B and Appendix Fig S1). Although expression of the mutant alleles was very low in the parental cells, expression of the maternal allele was elevated in the cells of patient 1 (hereafter called patient) and was responsible for the low expression of wild-type protein in these cells due to infrequent recognition of the original splice site (Fig 1C and Appendix Fig S1C).

CENATAC is an essential gene whose product has previously been reported to interact with pre-mRNA splicing factors and to localize to centrosomes where it suppresses centriole overduplication and spindle multipolarity (Hart *et al,* 2015; Wang *et al,* 2019). Analysis of *CENATAC* sequence conservation in metazoan species revealed the presence of two N-terminal C2H2 zinc fingers and four well-conserved C-terminal sequence motifs, of which the two most C-terminal ones are lost as a result of the patient mutations (Fig 1B and Appendix Fig S2).

CENATAC promotes error-free chromosome segregation

Live imaging of chromosome segregation in *CENATAC* mutant patient lymphoblasts stably expressing H2B-mNeon (Yost *et al,* 2017) revealed a mild chromosomal instability phenotype, consistent with the modest levels of aneuploidy in blood cells of these patients (Figs 1A and 2A). To examine whether *CENATAC* patient mutations cause chromosomal instability, we expressed mutant *CENATAC* alleles in HeLa cells in which the endogenous loci were modified to express AID-degron-tagged CENATAC (HeLa$^{EGFP\text{-}AID\text{-}CENATAC}$,



Appendix Fig S3) (Nishimura et al, 2009). Efficient depletion of CENATAC through a combination of siRNA treatment and auxin addition caused chromosome congression defects and a subsequent mitotic arrest (Fig 2B and C, and Appendix Fig S3). This phenotype was fully rescued upon re-expression of wild-type but not MVA mutant CENATAC (Fig 2C and Appendix Figs S4 and S5), indicating that both MVA mutants are defective for CENATACÕs function in mitotic chromosome congression. MVA mutant CENATAC caused a similar mitotic phenotype when expressed in near-diploid DLD-1 cells (Appendix Fig S6). CENATAC alleles missing either of the two most C-terminal conserved motifs that are absent from MVA mutant CENATAC (Fig 1B, motifs 3 and 4) did not rescue the mitotic defects. Instead, the expression of the MVA or motif 3/4 mutants exacerbated the phenotype, suggesting that these proteins dominantly repressed the function of any residual wild-type protein (Fig 2C). Mutations in the zinc fingers or deletion of motifs 1 or 2 only partly compromised CENATAC function (Figs 1B and 2C).

Live imaging of HeLa$^{EGFP\text{-}AID\text{-}CENATAC}$ cells with fluorescently labeled chromatin and microtubules revealed that the chromosome congression defect upon CENATAC depletion preceded the previously described loss of spindle bipolarity (Figs 2D and E, and EV2A and B, Movies EV1 and EV2) (Wang et al, 2019). In addition, we did not observe centriole over-duplication in CENATAC-depleted cells (Fig EV2C and D). This is in contrast to what was recently reported for CENATAC knockout cells (Wang et al, 2019), raising the possibility that centriole over-duplication is a cumulative effect of prolonged CENATAC loss. Our attempts to examine this failed, as we were unable to create CENATAC knockout cells, consistent with it being an essential human gene (Blomen et al, 2015; Hart et al, 2015; Wang et al, 2015). Taken together, these data show that CENATAC directly or indirectly promotes chromosome congression in mitosis (in a manner likely unrelated to its role in maintaining spindle bipolarity) and that MVA mutant CENATAC is a defective variant.

CENATAC is a novel component of the minor spliceosome

To investigate in which processes CENATAC plays a role, we performed a genome-wide, evolutionary co-occurrence analysis.

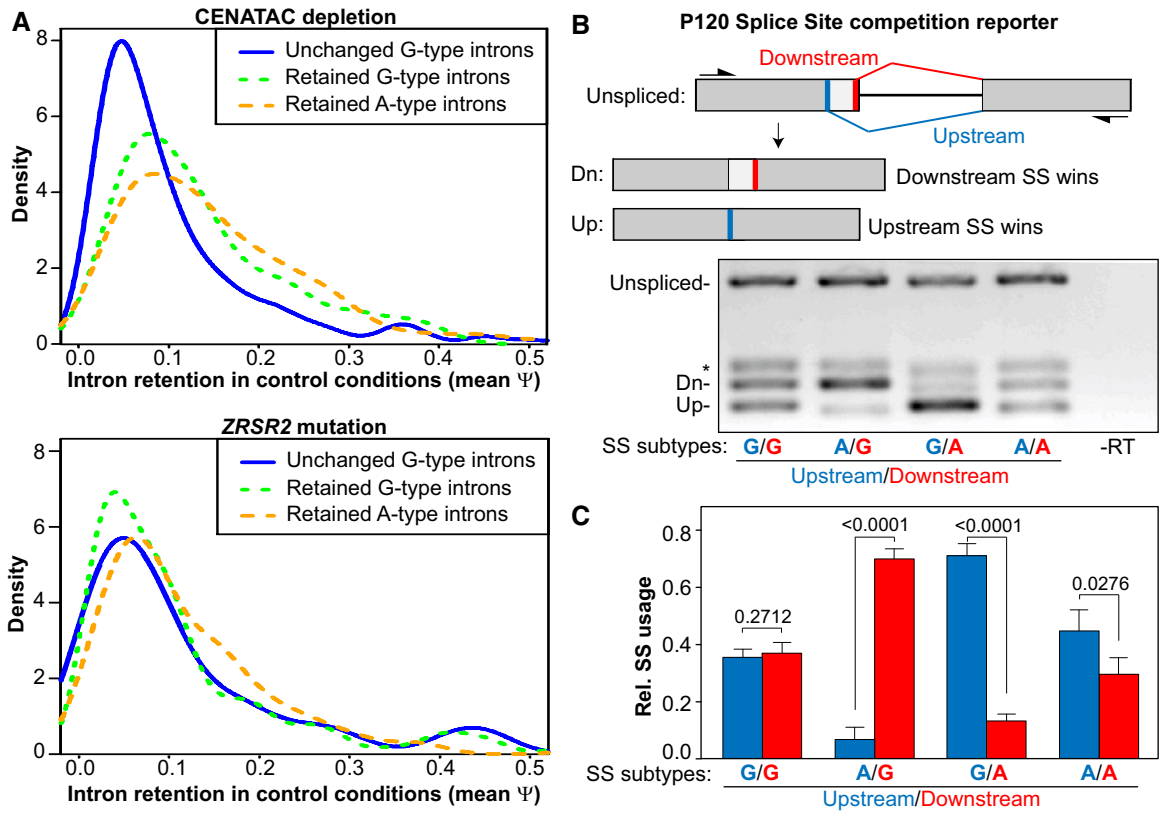


Figure 6. A-type minor introns are spliced less efficiently.

A Density plots showing intron retention (Ψ) values in the HeLa unedited parental control cell line (depleted of GAPDH) of A- and G-type minor introns that were either unchanged or retained after CENATAC depletion (top) or *ZRSR2* mutation (bottom). The median psi values of the retained G- and A-type introns are significantly higher (Psi = 0.130 and Psi = 0.115, respectively; $P < 0.01$, Mann–Whitney rank-sum test) compared with the unchanged introns (Psi = 0.081) in the CENATAC depletion dataset.

B RT–PCR P120 reporter assay (Hall & Padgett, 1996) to measure the relative usage of A-type (AT-AC) and G-type (GT-AG) 5′ splice sites in direct competition. Upper: schematic diagram showing the overall architecture of the reporter construct with its down- and upstream splice site (thick red and blue bars, respectively) and the products created by splicing (Dn and Up, respectively). Lower: RT–PCRs of the reporter with A- or G-type splice sites in the down- or upstream positions as indicated below the gel. SS, splice site. *PCR product after use of a cryptic major splice site (not shown in the schematic).

C Quantification of relative splice site usage of A- and G-type splice sites in (B) (three biological replicates).

Data information: In (C), data are presented as mean ± SEM. *P*-values were calculated with unpaired Student's *t*-tests. The CENATAC depletion and *ZRSR2* mutation (MDS) datasets (panel (A)) consist of three and eight biological replicates, respectively.

expression. CENATAC undergoes reversible modifications (acetylation and phosphorylation) (Wang *et al,* 2019), which may provide the means to regulate its activity (also) in the minor spliceosome.

Presently, all mutations associated with MVA have been mapped to genes that are known regulators of chromosome segregation. Our discovery of disease-causing mutations in *CENATAC* extends this list for the first time with a mRNA splicing factor. Although a recent study showed that CENATAC regulates centriole duplication (Wang *et al,* 2019), we were unable to verify this. Instead, our data argue that chromosomal instability by CENATAC malfunction may instead be the result of a primary defect in splicing of A-type minor introns. Nevertheless, it remains possible that CENATAC can also promote high-fidelity chromosome segregation more directly, as has been suggested for several other proteins involved in splicing (Montembault *et al,* 2007; Pellacani *et al,* 2018; Somma *et al,* 2020).

Strikingly, the clinical phenotype of *CENATAC* mutant MVA strongly resembles that of MOPD1/TALS, Roifman and Lowry–Wood syndromes, which are caused by mutations in the U4atac snRNA component of the minor spliceosome. Patients with these syndromes likewise present with microcephaly, developmental delay, and retinal abnormalities (Farach *et al,* 2018). No aneuploidies were reported (Hallermayr *et al,* 2018; Wang *et al,* 2018), but karyotype analyses were not performed for the majority of patients. It will therefore be of interest to examine whether aneuploidies occur in some of these patients, and whether (and to what extent) the affected transcripts and the splicing defect differ between MVA and these syndromes.

Although the depletion of both ZRSR2 and CENATAC caused a chromosome congression defect in mitosis (Fig 4), patients with *ZRSR2* mutations are clinically different from MVA patients with *CENATAC* mutations. This difference is most likely related to differences in their splice targets, such as the G-type minor introns that are differentially affected by depletion of CENATAC vs ZRSR2 (Fig 5). Mutations in *ZRSR2* are associated with MDS and clonal cytopenias of unknown significance (CCUS) (Madan *et al,* 2015; Fleischman *et al,* 2017). In line with ZRSR2's mitotic phenotype,

various stable aneuploidies were observed in MDS and CCUS patients with *ZRSR2* mutations (Madan *et al,* 2015; Fleischman *et al,* 2017; Hosono, 2019), though it is unclear whether mitotic defects contribute to these disease phenotypes. It would be of interest to investigate whether mitotic defects negatively impact erythropoiesis in these patients.

Materials and Methods

Samples

The MVA exome analyses were approved by the London Multicentre Research Ethics Committee (05/MRE02/17). Appropriate consent was obtained from patients and/or parents as applicable and the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report. DNA was extracted from whole blood using standard protocols. RNA was extracted from EBV-transformed lymphoblastoid cell lines (LCLs) using the RNeasy Mini Kit protocol (Qiagen).

For the functional experiments, the following patient LCLs were used: ID_5728_1 (patient, biallelic *CENATAC* (*CCDC84*) mutations, ECACC ID: FACT5728DLB), ID_5728_3 (sibling, no *CENATAC* mutations, ECACC ID: FACT5728KC), ID_5728_4 (father, monoallelic *CENATAC* mutation, ECACC ID: FACT5728GLB), and ID_5728_5 (mother, monoallelic *CENATAC* mutation, ECACC ID: FACT5728ALB).

Lymphoblastoid cell lines were cultured in RPMI supplemented with 15% fetal bovine serum (FBS), 100 μg/ml penicillin/streptomycin, and 2 mM alanyl glutamine. Cells expressing H2B-mNeon were created by lentiviral transduction, using standard procedures. Imaging of LCLs was performed as previously described (Yost *et al,* 2017).

Exome sequencing, alignment and variant calling, reference data sets, PTV prioritization method, recessive analysis, and Sanger sequencing: as previously described (Yost *et al,* 2017).

cDNA analysis of *CENATAC* (*CCDC84*) mutations

We synthesized cDNA using the ThermoScript RT–PCR System (Life Technologies) with random hexamers and 1 μg of total RNA. We amplified the mutation regions using cDNA-specific primers and sequenced the PCR products as described above. Primer sequences are available on request.

Conservation logos

Hidden Markov model (HMM) profiles were created from iterative jackhmmer searches (Potter *et al,* 2018) (version: HMMER3/f [3.1b2 | January 2014]) with CENATAC's protein sequence against the sequences of all metazoan species within the UniProt database. In-between successive iterations, non-CENATAC sequences were manually removed. Logos were created using Skylign (Wheeler *et al,* 2014); letter height: information content above background.

Immunoblots

For Western blot samples, cells were treated as indicated and lysed in Laemmli lysis buffer (4% SDS, 120 mM Tris pH 6.8, and 20% glycerol). Lysates were processed for SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Immunoblotting was performed using standard protocols. Visualization of signals was performed on an Amersham Imager 600 scanner using enhanced chemiluminescence. Primary antibodies used were rabbit anti-CENATAC (CCDC84; Sigma, HPA071715) and mouse anti-Tubulin (Sigma; T5168). Secondary antibodies used were goat anti-mouse HRP (170-6516) and goat anti-rabbit HRP (170-6515), both obtained from Bio-Rad.

Cell culture

HeLa T-REx Flp-In osTIR-9Myc::NEO cells (gift from Andrew Holland) were cultured in DMEM high glucose supplemented with 10% Tet-approved FBS, 100 μg/ml penicillin/streptomycin, and 2 mM alanyl glutamine. DLD-1 cells (ATCC CCL-221) were cultured in DMEM/F-12 supplemented with 10% Tet-approved FBS, 100 μg/ml penicillin/streptomycin, and 2 mM alanyl glutamine. HeLa S3 cells (a kind gift from Dr. Joan Steitz) were cultured in suspension in 1640 RPMI supplemented with 10% FBS, 2 mM glutamine, and 100 μg/ml penicillin/streptomycin. Stable expression of H2B-mNeon was done by lentiviral transduction using standard procedures. All cell lines were regularly tested and at all times found to be mycoplasma-free.

Creation of HeLa$^{EGFP\text{-}AID\text{-}CENATAC}$ and HeLa$^{EGFP\text{-}CENATAC}$ cell lines

HeLa$^{EGFP\text{-}AID\text{-}CENATAC}$ and HeLa$^{EGFP\text{-}CENATAC}$ cell lines were derived from HeLa T-REx Flp-In osTIR-9Myc::NEO and HeLa T-REx Flp-In, respectively. Tagging of the endogenous locus of CENATAC was done according to the scCRISPR protocol (Arbab *et al,* 2015) using the Protospacer, HDR_insert, and HDR_ext primers in Table EV3. pcDNA5-FRT-TO-EGFP-AID (Addgene, 80075) was used as template for both the EGFP-AID and EGFP tags. Cells were transfected with Lipofectamine LTX using standard procedures and subsequently FACS-sorted (single cells) based on EGFP expression. Endogenous tagging was confirmed by PCR (using the Genomic primers, Appendix Fig S3A) and immunoblotting of CENATAC protein (Appendix Fig S3B and C).

Viral plasmids, cloning, and viral production

For lentiviral re-expression of CENATAC variants, first pcDNA5 PURO FRT TO EGFP-AID-CENATAC was created by cloning CENATAC cDNA derived from HeLa cells into empty pcDNA5-FRT-TO-EGFP-AID (Addgene, 80075) using the cDNA PCR primers in Table EV3 and digestion of both the PCR product and the plasmid with NotI/ApaI. The CENATAC cDNA was subsequently cloned into pcDNA5 PURO FRT TO containing a LAP-tag to create pcDNA5 PURO FRT TO LAP-CENATAC by Gibson assembly (Gibson *et al,* 2009) with the PCR primers Gibson1 and Gibson2. Mutagenesis was then performed to make this construct resistant to CENATAC siRNA treatment (CCDC84; Dharmacon, J-027240-07) by Gibson assembly with PCR primers Gibson3. Next, in the siRNA-resistant construct, CENATAC wild-type cDNA was mutated to Mut1 (primers Gibson4), Mut2 (Gibson5), 2xZF (Gibson6; two consecutive rounds of cloning), Δ1 (Gibson7), Δ2 (Gibson8), Δ3 (Gibson9), or Δ4 (Gibson10) by Gibson assembly. Lentiviral CENATAC iresRFP constructs

were derived from a lentiviral construct encoding fluorescently tagged histone 2B (H2B) and a puromycin-resistant cassette (pLV-H2B-mNeon-ires-Puro) (Drost *et al,* 2015). First, the fluorescently tagged H2B was substituted by CENATAC derived from pcDNA5 PURO FRT TO LAP-CENATAC (see above) by Gibson assembly with PCR primers Gibson11 and digestion by AscI/NheI. Next, the puromycin-resistant cassette was substituted by tagRFP by Gibson assembly with PCR primers Gibson12. Finally, all siRNA-resistant variants of CENATAC were cloned from their respective pcDNA5 PURO FRT TO LAP-CENATAC plasmids into pLV CENATAC ires-tagRFP by Gibson assembly with PCR primers Gibson13 and PstI digestion of the plasmid. Virions were generated by transient transfection of HEK 293T cells with the transfer vector and separate plasmids that express Gag-Pol, Rev, Tat, and VSV-G. Supernatants were clarified by filtration.

Immunoprecipitation

For each sample, a full 10-cm plate of HeLa$^{EGFP\text{-}AID\text{-}CENATAC}$ cells was used, treated as indicated (Appendix Fig S3C). The cells were lysed in ice-cold lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 2% NP-40, 0.1% deoxycholate, proteasome inhibitors) and treated with benzonase for 15 min at 4°C. After centrifugation, the supernatant was incubated with beads (GFP-Trap, Chromotek) for 2.5 h at 4°C and washed three times with ice-cold lysis buffer. The samples were finally eluted in Laemmli sample buffer.

Live cell imaging analysis of mitotic fidelity

Lymphoblastoid cell lines were imaged as previously described (Yost *et al,* 2017). siRNA transfections (RNAiMAX, Thermo Fisher) in HeLa$^{EGFP\text{-}AID\text{-}CENATAC}$ (40 nM siRNA) and DLD-1 cells (50 nM siRNA) were done against CENATAC (CCDC84; Dharmacon, J-027240-07), GAPDH (Dharmacon, D-001830-01-05), or ZRSR2 (Sigma, SASI_Hs02_00338940). In the case of CENATAC depletion in HeLa$^{EGFP\text{-}AID\text{-}CENATAC}$ cells, transfections were done in the presence of 1 mM 3-indoleacetic acid (IAA) or ethanol (IAA vehicle) for 24 h in a 24-well plate before the cells were re-plated to eight-well ibidi μ-slides with 2 mM thymidine (for early S-phase synchronization) and 100 μl lentivirus for CENATAC re-expression. After 18 h, the cells were released from thymidine for 6 h and imaged in CO_2-independent medium in a heated chamber (37°C), while air-tight-sealed in the well plate with parafilm. These cells were therefore imaged ~ 48 h after siRNA-mediated knockdown of CENATAC and ~ 24 h after lentivirus addition. For CENATAC depletion and re-expression in DLD-1 cells, the lentivirus (150 μl) was immediately added together with the siRNA treatment (instead of 24 h later together with the 2 mM thymidine). These cells were therefore imaged ~ 48 h after siRNA-mediated knockdown of CENATAC and ~ 48 h after lentivirus addition. For the experiments in Figs 2D and EV2A, the cells were additionally incubated with 200 nM SiR-tubulin dye (Spirochrome) for 6 h prior to imaging to facilitate visualization of the mitotic spindle. For the depletion of ZRSR2 in both HeLa$^{EGFP\text{-}AID\text{-}CENATAC}$ and DLD-1 cells, the cells were re-plated to 8-well ibidi μ-slides with 2 mM thymidine 48 h (instead of 24 h) after transfection and therefore imaged ~ 72 h after siRNA-mediated knockdown of ZRSR2. Images were acquired every 3 or 5 min at 1 × 1 binning in 7 × 2.5 μm *z*-stacks (RFP as in Appendix Figs S4 and S6 was imaged in only 1 *z*-stack per position) and projected to a single layer by maximum intensity projection using NIS-Elements Software 4.45. Imaging was performed with a Nikon Ti-Eclipse wide-field microscope equipped with an Andor Zyla 4.2 sCMOS Camera, 40× oil objective NA 1.3 WD 0.2 mm, and Lumencor SPECTRA X light engine. Analysis of these experiments was carried out with ImageJ software. When applicable, cells re-expressing CENATAC variants were identified through co-expression of cytosolic RFP (via ires-tagRFP); RFP-negative cells were omitted from the quantifications (Appendix Figs S4 and S6).

Immunofluorescence imaging

After treating the cells with siRNAs and IAA (see above) for 24 h in a 24-well plate, the cells were re-plated on round 12-mm coverslips and treated with 2 mM thymidine (for early S-phase synchronization) for 24 h. 10 h after release, MG132 was added for 45 min after which the cells were pre-extracted with 0.1% Triton X-100 in PEM (100 mM PIPES pH 6.8, 1 mM $MgCl_2$, and 5 mM EGTA) for ± 60 s. After 60 s, 4% paraformaldehyde was added on top of the PEM in a 1:1 ratio (400 μl each) for 20 min to fixate the cells. The coverslips were subsequently washed twice with PBS and blocked with 3% BSA in PBS for 16 h at 4°C, incubated with primary antibodies for 2 h at room temperature, washed three times with PBS containing 0.1% Triton X-100, and incubated with secondary antibodies for 1 h at room temperature. Coverslips were then washed four times with PBS/0.1% Triton X-100 and mounted using ProLong Gold Antifade with DAPI (Molecular Probes). All images were acquired on a deconvolution system (DeltaVision Elite; Applied Precision/GE Healthcare) equipped with a 100×/1.40 NA UPlanSAPO objective (Olympus) using Softworx 6.0 software (Applied Precision/GE Healthcare). The images are maximum intensity projections of deconvoluted stacks. Random pro-metaphase and metaphase cells were selected, and centrioles were counted by hand. Primary antibodies used were rabbit anti-Centrin1 (Abcam, ab101332, 1/500) and mouse anti-Tubulin (Sigma, T5168, 1/10,000). Secondary antibodies used were goat anti-mouse 647 (A21236) and goat anti-rabbit 568 (A11036), both obtained from Thermo Fisher.

Co-evolution analysis

First, a phylogenetically diverse set of complete eukaryotic-predicted proteomes was utilized. This set was previously compiled to contain the protein sequences of 90 eukaryotic species (Hooff *et al,* 2017; preprint: van Wijk & Snel, 2020). These species were selected based on their representation of eukaryotic diversity. If available, we selected two species per clade and model organisms were preferred over other species. If multiple proteomes or proteomes of different strains were available, the most complete proteome was selected. When multiple splicing variants of a single gene were annotated, the longest protein was chosen. A unique protein identifier was assigned to each protein, consisting of four letters and six numbers. The letters combine the first letter of the genus name with the first three letters of the species name. The versions and sources of the selected proteomes can be found in Table EV1.

To define phylogenetic profiles for all human proteins, we determined automatic orthologous groups (OG) across the database using

information from PANTHER 9.0 (Mi *et al,* 2016). PANTHER 9.0 contains 85 genomes within total of 1,136,213 genes. Of these genes, 759,627 genes are in PANTHER families with phylogenetic trees, multiple sequence alignments, and HMM profiles. In total, there are 7,180 PANTHER families and 52,768 subfamilies. Families are groups of evolutionary-related proteins and subfamilies are related proteins that are likely to have the same function. The division into subfamilies is done manually, by biological experts. Every subfamily of PANTHER is an OG at some taxonomic level in the tree of life. We used "hmmscan" tool from the HMMER package (Potter *et al,* 2018) (HMMER 3.1b1) to find for each protein sequence in our database, the best matching profile of a main family or subfamily in PANTHER9.0. The phylogenetic profile of panther main or subfamily was subsequently defined by utilizing the hierarchical nature of the panther classification. Specifically, the phylogenetic profile of a main or subfamily also includes all members of daughter families (and if relevant their daughter families, etc.). Note that due to the automatic nature of orthology definition and the draft quality of a few genomes, phylogenetic profiles of the human proteins are not as accurate as those defined by manual analysis (van Hooff *et al,* 2019).

To determine the phylogenetic profile similarity, Pearson's correlation (https://en.wikipedia.org/wiki/Phi_coefficient) was computed between the phylogenetic profile of the CENATAC panther (PTHR31198) and the phylogenetic profile of all other panther sub- and main families using in-house scripts. To detect functional patterns in orthologous groups with similar phylogenetic profiles (correlation > 0.5), a GO enrichment analysis was performed (Ashburner *et al,* 2000; Carbon *et al,* 2019; Mi *et al,* 2019). GO cellular component overrepresentation (GO Ontology database: released 2020-01-03) was computed using PANTHER (test release 2019-07-11) with the human reference genome gene set as background. Statistical significance of overrepresented GO terms was computed using Fisher's exact test with FDR correction.

Nuclear extract and GFP pull-down and mass spectrometry

Nuclear extract of wild-type and HeLa$^{EGFP\text{-}CENATAC}$ cells was prepared as described earlier (Kloet *et al,* 2016). In short, cells were harvested by trypsinization and resuspended in cold hypotonic buffer (10 mM HEPES KOH pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl). Afterward, the cell pellet was homogenized using a Douncer with type B pestle (tight) to lyse the cell membrane. After centrifuging, the nuclei were washed with cold PBS and resuspended in cold buffer for lysis (420 mM NaCl, 20 mM HEPES KOH pH 7.9, 20% v/v glycerol, 2 mM $MgCl_2$, 0.2 mM EDTA) followed by rotation, centrifugation, and collection of the nuclear extract. 450 µl of nuclear extract was used for each GFP pull-down using 15 µl slurry of GFP-Trap agarose beads (Chromotek), performed in triplicate. GFP pull-downs were done as described earlier (Smits *et al,* 2013), without the addition of EtBr during the incubation, and with an adapted buffer C (150 mM NaCl, 20 mM HEPES KOH pH 7.9, 20 % v/v glycerol, 2 mM $MgCl_2$, 0.2 mM EDTA, complete protease inhibitors w/o EDTA, 0.5 mM DTT) for the incubation (+0.1% NP-40) and washes (+0.5% NP-40). Samples were digested using on-bead digestion with trypsin overnight (Hubner & Mann, 2011). The tryptic peptides were acidified with TFA and purified on C18 StageTips (Rappsilber *et al,* 2007).

After elution from the C18 StageTips, tryptic peptides were separated on an Easy-nLC 1000 (Thermo Scientific), connected online to a Q Exactive HF-X Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific), using an acetonitrile gradient of 7–30% for 48 min followed by washes of 50–90% acetonitrile, for 60 min of total data collection. Full scans were measured with a resolution of 120,000, and the top twenty most intense precursor ions were selected for fragmentation with a resolution of 15,000 and dynamic exclusion set at 30 s. Peptides were searched against the UniProt human proteome (downloaded June 2017) using MaxQuant (Cox & Mann, 2008) (version 1.6.0.1) with default settings, and iBAQ, LFQ, and match-between-runs enabled. Data analysis was done using Perseus (version 1.5.5.3), and the volcano plot and stoichiometry calculations were done as described earlier (Smits *et al,* 2013) using in-house-made scripts for R (version 3.6.1).

Nuclear extract preparations for Northern blots

Nuclear extract from HeLa S3 suspension cells was prepared according to the protocol described by Dignam *et al* (1983) using buffer D containing 50 mM KCL in the final dialysis step.

Immunoprecipitation and Northern blots

100 µl nuclear extract diluted in lysis buffer to a final volume of 200 µl was incubated with 2 µg of anti-CCDC84 antibody (SIGMA-HPA071715) overnight in the cold room with end-to-end rotation. The following day capture of antibody–antigen complexes was done using 50 µl of resuspended Protein G Dynabeads prepared according to manufacturer's instructions and incubated with the nuclear extract antibody samples for 2 h at 4°C. Beads were then washed four times with lysis buffer lacking protease and RNase inhibitors. RNA was eluted by proteinase K treatment, extracted once with phenol:chloroform:isoamyl alcohol (25:24:1; pH 4.8) followed by ethanol precipitation. RNA was dissolved in H_2O or 0.1X TE buffer.

Total volumes of 2 µl (input) and 5 µl (IP) RNA samples were separated on a 6% polyacrylamide–urea gel and analyzed by Northern blotting essentially as described by Tarn and Steitz (1996). Individual snRNAs were detected using ^{32}P 5′-end-labeled DNA or LNA oligonucleotides complementary to individual snRNAs. Northern blots were exposed to image plates and visualized using Typhoon FLA-9400 Scanner (GE Healthcare, USA) at 50-micron resolution. The data were quantified using AIDA Software (Raytest, Germany).

Glycerol gradient and ultracentrifugation

HeLa S3 nuclear extracts were preincubated for 0–20 min at +30°C in a buffer containing 13 mM HEPES (pH 7.9), 2.4 mM $MgCl_2$, 20 mM creatine phosphate, 2 mM DTT, 40 mM KCl, and 0.5 mM ATP. Aggregates were subsequently removed by a brief centrifugation (20,000 *g*, 1 min, +4°C), and the supernatant was subsequently ultracentrifuged on a linear 10–30% glycerol gradient (20 mM HEPES, pH 7.9; 40 mM KCl, 2 mM DTT, 2.4 mM $MgCl_2$) for 18 h at 29,000 rpm, +4°C, Sorvall TH641 rotor (RCF(max) = 143,915.6 *g*). Following ultracentrifugation, the samples were fractionated. 20% of each fraction was deproteinized and used for RNA isolation and Northern blotting and the remaining 80% was subjected to TCA precipitation, separated on a 10% SDS–PAGE, and analyzed by

Western blots. Each blot was probed for CENATAC (CCDC84-HPA071715; Sigma-Aldrich–Merck), PRPF4 (#HPA0221794, Sigma-Aldrich–Merck).

RT–PCRs

For Figs 4A and 4D, and Appendix Fig S10: Total cellular RNA was extracted using the RNeasy Kit Protocol (Qiagen) and treated with DNase I amplification grade (Invitrogen) to remove potential genomic DNA contamination. cDNA synthesis was carried out using SuperScript™ II RT (Thermo Fisher Scientific) and Oligo(dT)18 primers. PCRs were performed with Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific) with the following cycling conditions: initial denaturation (98°C for 60 s), followed by 28–30 cycles of denaturing (98°C for 10 s), annealing (gene-specific temp. for 30 s), extension (72°C for 15–20 s), and a final extension (72°C for 1 min 30 s). PCR primers and relevant annealing temperatures are listed in Table EV3. PCR products were analyzed on 2% agarose gel run using 1X TBE buffer. For Figs 4B and 5E, total RNA isolated was isolated from HeLa cells or patient/control subject lymphoblasts using TRIzol extraction followed by an additional acidic phenol (pH 5.0) extraction. 1 μg of RNA was converted to cDNA using maxima H minus reverse transcriptase (Thermo Fisher) according to the manufacturer's protocol. PCRs were performed essentially as described above, and gene-specific primers and annealing temperatures are listed in Table EV3.

RNA isolation and high-throughput sequencing

Total RNA isolated was isolated from HeLa$^{EGFP\text{-}AID\text{-}CENATAC}$ cells treated with siGAPDH (Dharmacon, D-001830-01-05) for 48 h or with siCENATAC (CCDC84, Dharmacon, J-027240-07) and 1 mM 3-indoleacetic acid (IAA) for 24 or 48 h, or unedited HeLa parental cells treated with siGAPDH for 48 h, or patient/control subject lymphoblasts using TRIzol extraction followed by an additional acidic phenol (pH 5.0) extraction. RNAseq libraries were constructed using Illumina TruSeq Stranded Total RNA Kit (Illumina) Human Ribo-Zero rRNA Depletion Kit (Illumina). Paired-end 150 + 150 bp sequencing was done with Illumina NextSeq 500/550 High Output Kit v2.5 for HeLa samples and with Illumina NovaSeq 6000 using partial S4 flow cell lane for patient samples.

Mapping the reads to the genome

The STAR aligner (Dobin *et al,* 2013) was used for mapping the paired sequence reads to the genome (hg38/GRCh38). Transcript annotations were obtained from GENCODE (v29). The length of genomic sequence flanking the annotated junctions (sjdbOverhang parameter) was set to 161. The Illumina adapter sequences AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC and AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCATT were, respectively, clipped from the 3′ of the first and the second pairs in the read libraries (using clip3pAdapterSeq parameter).

Differential alternative splicing analysis

Differential AS analysis was done using Whippet (v0.11) (Sterne-Weiler *et al,* 2018). Both merged aligned reads (bam files) and AS event annotations from GENCODE (v29) were used to build the index reference for AS events. To detect the significantly differential events, probability cutoff of Pr > 0.9 and percentage spliced in deviation cutoff of $|\Delta\Psi| > 0.1$ were used.

Differential intron retention analysis

For a comprehensive and sensitive IR analysis, the IntEREst R/Bioconductor package was used (Oghabian *et al,* 2018). After reading binary alignment (.bam) files, IntEREst detects introns with significantly higher and lower number of mapped reads relative to the number of reads that span the introns. The DESeq2-based function of IntEREst, i.e., deseqInterest(), was used for the differential IR analysis. The Benjamini–Hochberg method was used for adjusting the *P*-values, and a cutoff of $P_{adj} < 0.05$ was applied to extract the significantly differential IRs. The reference table was built from the NCBI RefSeq transcription annotations based on hg38/GRCh38 genome assembly.

Annotating minor introns

We used IntEREst R/Bioconductor package to annotate the minor (U12-type) introns as described previously (Oghabian *et al,* 2018) using threshold values of 0.07 and 0.14 for 5′ss and BPS scores, respectively. BPS was identified by scanning intronic region from position −40 to position −3 upstream of the 3′ss, and the highest scoring sequence was selected as the BPS. This list was manually appended with additional introns that did not fulfill our annotation criteria (typically because of poor BPS), but have been previously identified as minor introns (Chang *et al,* 2007).

P120 minigene cloning, transfection, and analysis of RNA

The double 5′ss constructs were created by insertion mutagenesis PCR using the P120 minigene (Hall & Padgett, 1996) as a template, and further modifications of 5′ splice sites were made by PCR using mutagenic primers (for a list of primers used see Table EV3). The 3′ss was modified to accommodate for GT-subtype splicing by insertion of a CAG trinucleotide sequence through insertion mutagenesis PCR. All mutations were confirmed by DNA sequencing. Chinese hamster ovary cells were transfected with the double 5′ss constructs (1,600 ng per well of a 12-well plate) using Lipofectamine 2000 (Thermo Fisher Scientific), and after 24 h, total RNA was isolated using TRIzol reagent (Thermo Fisher Scientific). Following DNase treatment, a pCB6 vector-specific oligonucleotide (ACAGGGATGCCA) was used for reverse transcription of the RNA with RevertAid (Thermo Fisher Scientific). RT–PCR was performed with primers binding exon 6 (GGATGAGGAACCATTTGTGC) and exon 7 (AGAACGAGACCGCCCTTC), and the resulting PCR products were analyzed on a 3% MetaPhor™ (Lonza) agarose gel. The gel was imaged using Fuji LAS-3000 CCD Camera, and the band intensities were quantified using AIDA Software (Raytest, Germany). Identities of the PCR products were confirmed by DNA sequencing.

Data availability

The authors declare that the data supporting the findings of this study are available within the paper and its supplementary

information. The ICR1000 UK exome series data are available at the European Genome-Phenome Archive (EGA), Reference Number EGAS00001000971 (https://ega-archive.org/studies/EGAS00001000971). Exome data for individual patients cannot be made publicly available for reasons of patient confidentiality. Qualified researchers may apply for access to these data, pending institutional review board approval.

HeLa$^{EGFP\text{-}AID\text{-}CENATAC}$ RNAseq data were deposited in Gene Expression Omnibus GSE143392 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE143392). RNAseq data from the patient and control subject cannot be made publicly available for reasons of patient confidentiality. Qualified researchers may apply for access to these data, pending institutional review board approval.

Protein interaction AP-MS data were deposited in PRIDE PXD024682 (https://www.ebi.ac.uk/pride/archive/projects/PXD024682).

Expanded View for this article is available online.

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Author contributions
BW, GJPLK, MJF, NR, and ECT conceptualized the data. BW, AO, MVA, SH, JEH, SY, ECT, LV, MPAB, ECHU, JV, LER, and PP investigated the data. BW, AO, MVA, SH, JEH, SY, ECT, LV, MPAB, ECHU, JV, LER, and PP involved in formal analysis. BW, AO, MVA, SH, JEH, SY, ECT, LV, MPAB, JV, PP, and MJF designed methodology. BW, AO, MVA, SH, JEH, SY, ECT, LV, MPAB, ECHU, JV, LER, and PP validated the data. BW, AO, MVA, SH, ECT, LV, JV, and LER visualized the data. BW, AO, MVA, SH, SY, LV, JV, and LER curated the data. AO and JEH provided software. BW, GJPLK, MJF, MVA, and NR wrote the original manuscript and prepared draft. BW, GJPLK, MJF, BS, MV, NR, BI, AO, MVA, SH, JEH, SY, ECT, LV, ECHU, JV, and LER wrote, reviewed, and edited the manuscript. GJPLK, MJF, BS, MV, NR, and BI administered the project. GJPLK, MJF, BS, MV, NR, and BI supervised the data. GJPLK, MJF, BS, MV, NR, and BI provided resources. GJPLK, MJF, BS, MV, NR, and BI acquired funding.

Conflict of interest
Nazneen Rahman is a non-executive director of AstraZeneca. The other authors declare no competing interests.

Materials and correspondence

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Geert Kops (g.kops@hubrecht.eu).

Note

[1]CENATAC was named after AT-AC introns, which make up 84% of U12 A-type introns

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